STUDIES WITH THE AGAR CUP-PLATE METHOD
I. A STANDARDIZED AGAR CUP-PLATE TECHNIQUE

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Official Circular 198 (Ruehle and Brewer, 1931) gives directions for performing the agar cup-plate test. However, in the limited literature dealing with the subject, no mention is made of the influence of varying the volume of agar, the amount of inoculum, the hydrogen ion concentration or other factors. In the course of other studies with the agar cup-plate method (Rose and Miller, 1939; Miller and Rose, 1939) it was learned that a slight modification in procedure caused a considerable difference in the experimental results. It therefore became necessary to study the influence of variations in technique in greater detail. The object was to standardize the method so that more constant data could be obtained. The present communication deals with this study.

TECHNIQUE

A simple device is used to remove the disc of agar from the medium. This instrument[1] consists of a thin-walled stainless steel cylindrical chamber measuring 2.5 cm. in length and having a diameter of 1.5 cm.; the cutting edge is bevelled on the inside. A capillary metal tube, about 10 cm. long, is attached to the bottom of the cylindrical chamber. The chamber is sterilized by dipping in alcohol and flaming. Placing the open end of the chamber on the surface of a poured agar plate, the disc is cut easily with slight pressure. A finger is then placed over the tip

[1] Made by Mr. Charles Simpson, Laboratories of the Philadelphia General Hospital.

of the capillary tube, as in pipetting; the disc of agar is gently pulled slightly to one side and removed. With practice, no cracks are made in the walls of the cup.

The ordinary inoculating loop is not entirely satisfactory for removing small pieces of agar for subculture. A micro-spoon[2] is convenient for such purposes. The spoon is formed at the end of a piece of 10 per cent iridium-platinum No. 19 B. & S. gauge wire, 4 cm. long; it measures 2 mm. in diameter and has a shallow concavity approximately 0.75 mm. deep. The handle is mounted on a standard needle holder. About 30 seconds are required to cool the micro-spoon after sterilization in an open flame.

When it is necessary to determine whether the zone of inhibition of bacterial growth is bactericidal or bacteriostatic, a small piece of agar is removed with the micro-spoon, transferred to 10 ml. of broth, and incubated at 37°C. Readings are made at the end of 48 hours. Growth in a subculture tube indicates inhibitory rather than germicidal action of the antiseptic.

The antiseptics used in this study were limited to a single member of each of three groups. Carbolic acid was chosen for the phenol group because it has long been used as a standard. The dilution of 1:20 was employed because this was the weakest dilution which produced a zone free of bacterial growth of approximately 3 mm.; this size was found to be a convenient minimal one for accurate measurement. Mercury bichloride and crystal violet,[3] each in 1:1,000 dilution, were taken as representatives of the heavy metals and antiseptic dyes respectively. In one instance, neutral acriflavine[3] was added, to illustrate further the effect of the hydrogen ion concentration of the medium on the size of the zone. All hydrogen-ion-concentration determinations were made colorimetrically.

Except where experimental conditions required a change in procedure, the following technique was employed. Beef extract agar (Ruehle and Brewer, 1931) was kept melted at 48°C. in a con-

[2] Made by J. Bishop and Company, Platinum Works, Malvern, Penna.

[3] Supplied through the courtesy of National Aniline and Chemical Company, New York, N. Y.

stant temperature incubator. Each 30 ml. amount of 1.5 per cent extract agar at pH 6.8 was inoculated with 0.1 ml. of an 18-hour growth of the organism being studied. The cultures were standardized to a density of 1 billion organisms per milliliter with a barium sulphate nephelometer. Unless otherwise specified, our WP3 strain of *Staphylococcus aureus* was employed. As soon as the bacteria were added, the container was rotated to insure uniform distribution of organisms. Approximately 30 ml. amounts were poured into standard 100 x 15 mm. petri dish bottoms. The dishes were covered with Coors porcelain lids glazed on the outside. The porcelain lids absorbed water of condensation and prevented the deposition of moisture on the test medium. When the agar solidified, one disc of agar was usually cut from each plate. At times it was desirable for comparative purposes to cut three or four agar cups in a single dish. The standard volume of antiseptic employed was 0.2 ml. of a given dilution. The agar cup-plates were incubated in an upright position at 37°C. for 24 hours and readings were then made. All tests were performed in duplicate.

EXPERIMENTAL

Experiment 1. The amount of agar that has been recommended (Ruehle and Brewer, 1931) for the agar cup-plate test is 15 to 20 ml. However, when pouring plates, it is rather difficult to gauge the precise amount delivered. Furthermore, it was soon learned that a deep cup minimized the danger of spilling the antiseptic over the surface of the medium. As no data on the relationship of medium thickness to zone size was available, the question was answered as follows: A known quantity (usually 180 ml.) of 1.5 per cent agar was inoculated with 0.1 ml. of *Staphylococcus aureus* culture for each 30 ml. of medium. Varying quantities of agar from this one batch were pipetted into petri dishes and 0.2 ml. amounts of each antiseptic were placed in the cups. Uniform results were obtained when the agar volume was 25 to 35 ml. (table 1). We chose the 30 ml. amount as this produced a well of satisfactory depth.

Experiment 2. The sensitivity of the agar cup-plate method

TABLE 1

Influence of thickness of medium on zone size

ANTISEPTIC	AGAR	ZONE
	ml.	*mm.*
Phenol (1:20)	15	6
	25	3+
	30	3+
	35	3+
Mercury bichloride (1:1000)	15	11
	25	11
	30	11
	35	11
Crystal violet (1:1000)	15	8
	25	8
	30	8
	35	8

TABLE 2

Variation in the number of organisms

ANTISEPTIC	STAPH. AUREUS	ZONE
	ml.	*mm.*
Phenol (1:20)	0.1	3+
	0.2	3+
	0.3	3+
	0.4	3
	0.5	3
	1.0	2+
Mercury bichloride (1:1000)	0.1	11
	0.2	10+
	0.3	10
	0.4	10
	0.5	9
	1.0	8+
Crystal violet (1:1000)	0.1	8+
	0.2	8
	0.3	7
	0.4	6+
	0.5	6+
	1.0	6

to various numbers of bacteria was determined. An 18-hour growth of *Staphylococcus aureus* was standardized to one billion organisms per milliliter and amounts from 0.1 to 1 ml. were employed in the tests. It is evident that slight variations in the number of organisms (table 2) do not produce striking differences in the zone of inhibition of growth. One-tenth of a milliliter of a suspension of one billion organisms per milliliter usually yielded

TABLE 3

Varying the amount of antiseptic

ANTISEPTIC	VOLUME	ZONE
	ml.	*mm.*
Phenol (1:20)	0.1	2+
	0.2	3+
	0.3	4+
	0.4	6
	0.5	7
Mercury bichloride (1:1000)	0.1	11
	0.2	11+
	0.3	12
	0.4	12
	0.5	12
Crystal violet (1:1000)	0.1	7
	0.2	8
	0.3	8+
	0.4	8
	0.5	8+

a readily discernible line of demarcation between the zone of inhibition and the growth of bacteria. This amount of culture was taken as a standard in all subsequent studies.

Experiment 3. In order to test the influence of the amount of antiseptic on zone size, different amounts of a constant dilution of each antiseptic were placed in agar cups. The use of a graduated 1 ml. pipette gave more uniform results than the advocated "six drops." Although a drop more or less of antiseptic ordinarily does not cause marked changes in the results (table 3) it appears desirable to use a constant volume of a given dilution of anti-

septic in comparative studies. It was found that 0.2 ml. amounts were satisfactory.

Experiment 4. Different agar concentrations may yield varying results with some antiseptics (table 4). This difficulty may be obviated by using 1.5 per cent agar routinely. Our stock agar was usually prepared in a 3 per cent concentration. This was done in order to permit dilution up to 50 per cent with blood or serum. If an amount of protein solution less than 50 per cent were needed, saline or a saline-protein mixture was added to bring the agar dilution to 1.5 per cent. In another experiment with

TABLE 4

Effect of varying agar concentration

ANTISEPTIC	AGAR	ZONE
	per cent	*mm.*
Phenol (1:20)	3.0	3+
	2.0	3+
	1.5	3+
Mercury bichloride (1:1000)	3.0	11
	2.0	11
	1.5	11
Crystal violet (1:1000)	3.0	6+
	2.0	7+
	1.5	8

Staphylococcus aureus, it was found that a 50 per cent dilution of 3 per cent agar with saline gave zones identical in size with those obtained when an original 1.5 per cent agar was used.

Experiment 5. Although a difference in medium does not cause a marked change with phenol and crystal violet, the zone size with mercury bichloride is practically 50 per cent larger in extract agar than in beef infusion agar (table 5). This difference in results is probably attributable to the different amounts of protein in the media.

Experiment 6. To check the results obtained in Experiment 5, horse serum was added to extract agar to yield a 10 per cent serum concentration. Material from the same batch of plain extract

agar was used as a control. The comparative zones are shown in table 6; the effect of added protein is obvious.

Experiment 7. The size of the zones produced by phenol and crystal violet remained unchanged at three pH levels (table 7).

TABLE 5

Comparison of infusion with extract agar

ANTISEPTIC	ZONE	
	Extract agar	Infusion agar
	mm.	*mm.*
Phenol (1:20)	3+	3
Mercury bichloride (1:1000)	11	7
Crystal violet (1:1000)	8	7

TABLE 6

Influence of added serum

ANTISEPTIC	ZONE	
	Plain agar	10% serum agar
	mm.	*mm.*
Phenol (1:20)	3+	2+
Mercury bichloride (1:1000)	11+	6
Crystal violet (1:1000)	8	5+

TABLE 7

Hydrogen ion concentration of the medium

ANTISEPTIC	ZONE		
	pH 6.0	pH 7.0	pH 8.0
	mm.	*mm.*	*mm.*
Phenol (1:20)	3+	3+	3+
Mercury bichloride (1:1000)	14	11	10
Crystal violet (1:1000)	9	9	9
Acriflavine (1:1000)	4	5	6

On the other hand, with increasing alkalinity, the mercury compound showed a decrease in zone size while the dye, neutral acriflavine, revealed a definite increase in potency. This experiment emphasizes the importance of controlling and designating the

hydrogen-ion concentration of the medium in the agar cup-plate method.

Experiment 8. A virulent culture of *Staphylococcus aureus* (strain WP3) was selected for our tests. This organism grows homogeneously in broth and has a more golden color than the official F.D.A. strain but conforms with the F.D.A. requirements for the phenol coefficient test. The two strains gave the same size zone with the three antiseptics employed, (table 8) while

TABLE 8

Influence of bacterial strain

ANTISEPTIC	STAPH. AUREUS	ZONE
		mm.
Phenol (1:20)	F.D.A.	3+
	WP3	3+
	Smith	4
	HB-40	4
	W	4+
Mercury bichloride (1:1000)	F.D.A.	11
	WP3	11
	Smith	10
	HB-40	12
	W	12+
Crystal violet (1:1000)	F.D.A.	8
	WP3	8
	Smith	7+
	HB-40	7+
	W	8

other strains tested failed to give uniformly comparable zones of inhibition of growth. Obviously, it is important to use the official strain of *Staphylococcus aureus* or one that has been shown by actual test to have the same phenol resistance.

Experiment 9. Eight different bacteria, studied under identical experimental conditions, yielded a wide variety of zone size (table 9). It is clear that the test organism must be designated when comparative studies are made with the agar cup-plate method.

Experiment 10. The official phenol coefficient test requires the daily transfer of a standard loopful of culture into 10 ml. of broth for five consecutive days before doing the test. The question arose whether a similar technique was necessary for the agar cup-plate method. Accordingly, the WP3 *Staphylococcus aureus* was subcultured daily for five days in broth. The first subculture

TABLE 9

Data obtained with various organisms

ORGANISM	ZONE		
	Phenol (1:20)	Mercury bichloride (1:1000)	Crystal violet (1:1000)
	mm.	*mm.*	*mm.*
Staph. albus	1+	6+	7
Staph. aureus (WP3)	3	7	7
Strep. hemolyticus	7	7+	5
Pneumococcus (Type 1)	9	7	7
B. diphtheriae	8	4+	8
B. subtilis	3+	8	6+
B. typhosus	3	4	1+
B. coli	3+	3+	1

Beef infusion agar, pH 7.6, was used for all media in this experiment.

TABLE 10

Daily transfers versus 18-hour growths

ANTISEPTIC	ZONE	
	5-day transfer	18-hour growth
	mm.	*mm.*
Phenol (1:20)	3+	3+
Mercury bichloride (1:1000)	11+	11+
Crystal violet (1:1000)	8+	8+

was from our stock culture kept on an extract agar slant at room temperature and subcultured to agar at least once a month. The same organism was transferred from the stock culture to 25 ml. of broth and grown for 18 hours. The results of the tests with the two cultures are shown in table 10. The 18-hour growth, adjusted nephelometrically before using, yielded zones similar

in size to those produced by a *Staphylococcus aureus* culture which had been subcultured daily for five days.

Experiment 11. A parallel series of agar cup-plates was studied at three temperature levels: 37°C., 34°C., and 24.5°C. A variation in temperature causes a difference in the results obtained (table 11). A temperature of 37°C. was selected as a convenient standard for our tests.

Experiment 12. Under ordinary working conditions, bacteria are added to the agar, plates are poured, agar cooled, cups cut, an antiseptic added at once, and the preparation incubated at

TABLE 11

Influence of temperature of incubation

ANTISEPTIC	TEMPERATURE	ZONE
	°C.	mm.
Phenol (1:20)	37	3
	34	3+
	24.5	4+
Mercury bichloride (1:1000)	37	11
	34	11
	24.5	15
Crystal violet (1:1000)	37	8
	34	8+
	24.5	9

37°C. However, when many antiseptics are being tested simultaneously, a moderate interval of time may elapse between the pouring of the seeded agar and the addition of the antiseptic. Although this experiment shows that a lapse of five hours at room (24.5°C.) or refrigerator (14.5°C.) temperature has no obvious influence on the results (table 12), it is probably desirable to have the test plates ready for incubation within an hour after seeding.

On the basis of the data obtained in this study with three types of antiseptics, the following technique is suggested for the agar cup-plate method:

1. Use a "standard" organism. If *Staphylococcus aureus* is the test organism, use either the F.D.A. strain or one known to possess the same resistance to phenol.

2. Grow the bacteria for 18 to 24 hours in broth and standardize the suspension to one billion organisms per milliliter.

3. Extract agar, pH 6.8, may be employed. Some bacteria require the use of beef infusion agar, pH 7.4. Both types of medium are satisfactory, but the medium as well as the pH must be designated.

4. The final agar concentration should be 1.5 per cent. A 3 per cent agar, diluted to 1.5 per cent agar with saline, is satisfactory. Such an agar can also be reduced in concentration with protein (serum, blood) or protein-saline mixtures. In this manner protein-agar mixtures containing up to 50 per cent protein solution may be conveniently made for special studies.

TABLE 12

Time and temperature factors before addition of antiseptic

ANTISEPTIC	LAPSE OF TIME BEFORE ADDING ANTISEPTIC		
	0 hour	5 hours, 24.5°C.	5 hours, 14.5°C.
Phenol (1:20)	3+	3+	3+
Mercury bichloride (1:1000)	11+	12	11
Crystal violet (1:1000)	8	8	8

5. Seed the agar medium on the basis of 0.1 ml. of the standard suspension of organisms for each 30 ml. volume of medium. The temperature of the agar should be about 48°C., that is, the temperature ordinarily used in making blood agar medium.

6. Pour approximately 30 ml. of seeded agar medium into a standard 100 x 15 mm. petri dish bottom. Cover the petri dish with a Coors porcelain lid glazed on the outside.

7. After the agar solidifies, remove a disc of agar measuring 1.5 cm. in diameter. In some instances it is feasible and desirable to prepare three or more cups from a single plate.

8. Prepare appropriate dilutions of the antiseptic. Using a graduated 1 ml. pipette, place 0.2 ml. of a given dilution in each cup.

9. The antiseptic should be added and the plates should be ready for incubation within one hour from the time the bacteria are added to the agar medium.

10. Incubate test plates upright at 37°C. for 24 hours and then measure the width of the zone of inhibition of bacterial growth with a transparent ruler. The use of a hand lens in measuring the zones is an aid in obtaining accurate results.

Experiment 13. The technical procedure outlined above was checked as follows: Fresh solutions of the three antiseptics used

TABLE 13

Consistency of results obtained with the agar cup-plate method

DATE	ANTISEPTIC	ZONE	
		Plain agar	10% horse serum agar
		mm.	*mm.*
6/11	Phenol (1:20)	3	2+
6/12		3+	2
6/15		3+	2+
6/16		3+	2+
6/17		3+	2+
6/18		3+	2+
6/19		3+	2
6/11	Mercury bichloride	10+	5+
6/12	(1:1000)	11+	6
6/15		10+	6
6/16		11+	6
6/17		11+	6
6/18		11	6
6/19		11	5+
6/11	Crystal violet	8	5+
6/12	(1:1000)	7+	6
6/15		7+	5+
6/16		8	6
6/17		8	5+
6/18		8	5+
6/19		8	5+

in this study were prepared. The WP3 strain of *Staphylococcus aureus* was employed. Tests were then performed in duplicate using plain extract agar and extract agar containing 10 per cent horse serum. The same technique was followed on six other days and the readings obtained each day were recorded in table 13. An examination of this table shows that comparatively consistent

results can be obtained when all the technical requirements of the test are observed.

Other studies pertaining to the agar cup-plate method will be reported in separate communications (Rose and Miller, 1939; Miller and Rose, 1939).

CONCLUSIONS

A study was made of the agar cup-plate method of testing antiseptics. It was found that various factors may influence the results. Consequently, a standardized technique was developed. This technique is comparatively simple and yields consistent results with three representative antiseptics: phenol, mercury bichloride, and crystal violet.

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